New and Notable

FCS Imaging—A Way to Look at Cellular Processes

Zygmunt (Karol) Gryczynski
Center for Commercialization of Fluorescence Technologies, Department of Molecular Biology and Immunology, UNTHSC, Fort Worth, Texas

Fluorescence correlation spectroscopy (FCS) was introduced more than 30 years ago (1–3), but recent advances in optics, electronics, and biophotonics have enabled new FCS-based uses of quantitative analysis in the study of physiological processes including protein association and transport at the cellular level. Because of its high sensitivity and spectral selectivity, fluorescence fluctuation analysis becomes a very attractive and relatively easy approach that utilizes single molecule sensitivity in studies of biological processes on a subcellular level. In this issue, Digman et al. present a novel and interesting computational approach that enables robust Number and Brightness (N&B) image analysis. The N&B approach allows determining localized particle aggregation relevant for physiological function directly at the cellular level. This vigorous, but easy to implement and use method, provides brightness maps that reveal binding dynamics of focal adhesions and depict molecular aggregation in the cellular compartments.

As confocal microscopy reaches down to a single molecule detection level, the prominent features of spontaneous signal fluctuation become visible, revealing the fundamental basis of a biophysical system. Already, original works (1–4) distinguish that the fluctuating quantity is directly related to the number of solute particles of a particular species occupying a well-defined volume. The fundamental theorem connects fluctuation amplitudes with molecular concentrations and fluctuation relaxation spectra with the macroscopic transport coefficient. More importantly, observed fluctuations reveal macromolecular dynamics under equilibrium conditions without perturbation and are well suited for studying diffusion, flow, aggregation, and chemical reactions in the membrane, cellular components, and the whole cell.

The distribution of the fluorescence intensity fluctuations emitted from a small illumination volume depends, in a complex manner, on the quantum yield of the fluorophore and the number densities of the fluorescent species in the sample. This imposes the basic limitation that experimentally measurable stochastic fluctuations of fluorescence intensity in biologically relevant conditions (high concentrations) can only be observed for very small sample volumes. Unfortunately, basic principles of optics force relatively large limits for inherent resolution of confocal microscopy to $\sim\lambda/2$ ($\sim$200 nm for visible light), thus setting the minimum detection volume to a fraction of femtoliter (1 fl = 10–15 l). Although this appears to be a miniscule volume, a fraction of femtoliter is too large and only a low sample concentration can be used. Recently a number of different technologies have been introduced to reduce the detection volume below the diffraction limits. One group of approaches utilizes optical phenomena such as multiphoton-stimulated fluorescence microscopy (5,6) or stimulated emission depletion (7). A second group relies on the use of near-field phenomena like near-field scanning optical microscopy (8), zero-mode waveguide (9,10), confocal total internal reflection microscopy (11,12), and surface plasmon-coupled emission (13). All these approaches limit the detection volume to measure fluorescence signal fluctuations for higher concentrations which are more relevant for physiological conditions.

Another important challenge and limitation for applications of FCS to cellular imaging is the need for robust and fast data analysis. Most of the proposed methods are comparable to the ''moment analysis'' method originally proposed by Qian and Elson (14). These methods are sufficient for homogenous samples composed of a single molecular species and provide two quantities of interest: the number and the brightness of the studied molecules. Distinguishing multiple and diverse species resulting from protein interactions (association/aggregation) requires higher order momentums which drastically increase the complexity of data analysis. A more recent and general attempt, such as an approach based on photon-counting histogram analysis (15), considers the entire distribution of photon counts in a given volume. These approaches, however, generally require a large number of observations and are computationally too demanding to be applied for real-time, whole cell imaging.

Why would one like to have an instantaneous FCS image of the entire cell?

It is now well accepted that protein-protein interactions and protein conformational changes are the principle factors dictating all fundamental cell functions. For example, basic processes such as the flow of genetic information from DNA to RNA to protein, involve sequences of multiple and complex macromolecular interactions. All these processes are well organized and compartmentalized. Protein concentrations and aggregations not only differ in various locations in the cell, but may also quickly change during biological function. For example, the plasma membrane is a system with a well-defined structure that facilitates many life essential biochemical processes. Macromolecular motions in the membrane are well regulated and restricted, definitely not resembling free spontaneous diffusion in a solution. The local distribution, concentration, and interaction of different proteins will change during the course of physiological functions. A direct demonstration of protein

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Address reprint requests to Zygmunt Gryczynski, E-mail: zgryczyn@hsc.unt.edu.

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colocalization and/or aggregation during different stages of a biological process will be fundamental to our understanding of many regulatory mechanisms.

A persistent problem of FCS imaging is the relatively large observation volume and consequently significant background that complicates data analysis. The typical thickness of a membrane ($<$10 nm) is much below optical resolution, and observation of membrane-bound molecules will typically be compromised by the steady background of free diffusing fluorescent molecules. Commonly used two-photon scanning microscopy, the most effective approach we can use today for living cell imaging, is still heavily influenced by the presence of an immobile fraction either from intrinsic cellular features, background fluorescence, or slow intensity change due to photobleaching.

In this context, the contribution of Digman et al. in this issue provides an important step toward developing a versatile method that corrects for the variance of immobile fraction and autofluorescence providing maps of dynamic processes occurring at the cellular level. The N&B method is a computational approach that does not require any special hardware and can be applied with FCS systems in use today, including the spinning-disk confocal method. There are many important problems in cell biology that will rapidly benefit from this technique. The simplicity of the FCS measurements already stimulated efforts toward new biotechnology applications (16). The method presented by Digman et al. enables fast image analysis and can quickly be utilized for new analytical and diagnostic applications in a high throughput format.

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